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Changes in the Tibial Growth Plates of Chickens with Thiram-induced Dyschondroplasia

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Summary

Tibial dyschondroplasia (TD) is a metabolic cartilage disease of young poultry in which endochondral bone formation is disrupted leading to the retention of a non-calcified, avascular plug of cartilage in the tibial growth plate. Chicks aged 7 days were fed either a control diet or one containing thiram 100 ppm for 48 h to induce TD. Cell multiplication in the growth plate was determined thereafter with bromodeoxyuridine (BrdU) labelling, and metabolic changes by measuring alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP), and glutathione (GSH) activities. The effect on chondrocyte maturation was examined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of gene expression. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) and DNA fragmentation were used to determine the effects of thiram on cell survival. The results showed that thiram-induced TD was not due to the multiplication of cells in the post-proliferative zones. Thiram did not affect ALP activity, which would have indicated a loss of calcification potential, but it reduced both TRAP and the glutathione concentrations, suggesting that the growth plate metabolism and remodelling functions were adversely affected. Thiram appeared to have no effect on the expression of type X collagen, transglutaminase, RUNX2, or matrix metalloproteinase-2 (MMP) genes suggesting that it did not alter the maturation potential of chondrocytes. On the contrary, the expressions of MMP-13 and vascular endothelial growth factor (VEGF) genes were "up-regulated," suggesting that thiram has pro-angiogenic activity. However, TUNEL assay showed that thiram induced endothelial cell apoptosis in the capillary vessels of the growth plates, as early as 10 days of age, when TD was not visually evident. The vascular death increased on subsequent days accompanied by massive death of chondrocytes in the transition zone of the growth plate. The induction of apoptosis in the growth plate was also demonstrated by DNA fragmentation. It was concluded that thiram induced TD not through an increase in the multiplication of chondrocytes in the transition zone and not by altering the expression of genes causing the arrest of chondrocytes in a prehypertrophic state, but by creating a metabolic dysfunction which led to the destruction of blood capillaries in the transition zone chondrocytes.

Keywords: apoptosis; chicken; chondrocyte; thiram; tibial dyschondroplasia

Introduction

Tibial dyschondroplasia (TD) is a major metabolic cartilage disease in young poultry in which parts of the growth plate fail to undergo osteogenic transition leading to the retention of a thickened plug of avascular cartilage at the end of the proximal tibia and tibio-tarsal bones (Leach and Nesheim, 1965; Praul *et al.*, 2000; Farquharson, 2002). It is a leading cause of lameness in meat-type poultry due to growth plate fracture, infection, and bone deformation (Lynch *et al.*, 1992). The aetiology of TD is unknown and the pathogenesis of the disease poorly understood. However, studies

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of cellular and biochemical changes in lesions have demonstrated the "down-regulation" of many biomarkers associated with growth plate development and maturation (Gay and Leach, 1985; Lawler *et al.*, 1985; Thorp *et al.*, 1995; Wardale and Duance, 1996; Rath *et al.*, 1997; Pines *et al.*, 1998; Praul *et al.*, 2000; Farquharson, 2002). Since growth plate development depends on various growth and nutritional factors (Kronenberg, 2003; van der Eerden *et al.*, 2003), it has been suggested that the local or systemic imbalance of such factors prevents maturation of post-proliferative chondrocytes and subsequent osteogenesis (Praul *et al.*, 2000; Farquharson, 2002). Rath *et al.* (1994, 1998) demonstrated that many chondrocytes in TD-affected growth plates were non-viable and apoptotic, suggesting that the down-regulation of biochemical and molecular markers associated with maturation and hypertrophy was secondary to cell death.

Pathogenic studies in naturally occurring TD have the disadvantage that changes cannot be linked to the duration of the disease because of the unknown time of initiation. To circumvent this problem, Rath *et al.* (2004) modified an experimental model of TD induced by dithiocarbamate fungicides such as thiram and disulphiram (Vargas *et al.*, 1983; Edwards, 1984), by the use of a short thiram-feeding procedure. The present study describes the use of this procedure to examine the effects on growth plate development in respect of chondrocyte multiplication, metabolism and survival.

Materials and Methods

Chickens

Male broiler chicks (Cobb-Vantress, Fayetteville, AR, USA) were raised in Petersime batteries at a density of 12–13 birds/cage under a constant schedule of 23 h light and 1 h darkness. Chick starter diet, prepared according to National Research Council (1994) specifications, and water were provided *ad libitum*. The experiments accorded with the institutional animal care regulations.

Induction and Evaluation of TD

TD was induced as described by Rath *et al.* (2004). Chicks aged 7 days were deprived of feed overnight for 12–14 h before they were given either (1) a regular diet (control group), or (2) the same diet containing tetramethylthiuram disulphide (thiram) 100 ppm for 48 h, followed by the regular diet (experimental group). After the cessation of thiram administration, birds were killed by CO_2 inhalation at various timepoints up to 22 days of age to harvest growth plates for different assays. Routinely, 15–25 chickens in each group were kept until at least 15 days of age to determine the TD index (see below) and thus to ascertain the success of TD induction. At necropsy, the proximal growth plates of tibia were shaved longitudinally to determine the incidence and severity of TD. The severity was arbitrarily scored as 0 (growth plate normal), 1 (growth plate up to twice normal size), or 2 (growth plate more than twice normal size), as described by Rath *et al.* (2004). Most left over birds were killed on day 16 of age to assess the percentage and score the severity. The TD index of the growth plates was determined as the incidence multiplied by the severity score. Representative growth plates from eight birds in each group were fixed in neutral buffered formalin for histology and histochemistry. Some sections of growth plates were treated with KOH and stained with Alizarin red to examine for mineralization (Potthoff, 1984). Sections (5 μm) of undecalcified growth plates were stained with haematoxylin and eosin for light microscopy.

Tissue Harvest

For biochemical and gene expression studies chickens from both control and thiram-fed groups were killed on different days after feeding to harvest growth plate cartilage. At necropsy, longitudinal wedges of tibial growth plates were aseptically harvested with curettes (1- to 4-mm), freed of metaphyseal spongiosa, and placed in an appropriate incubation medium, extraction reagent, or tissue fixative as necessary. Because preliminary experiments showed a diminished yield of RNA in tissues with visually evident TD, most biochemical and gene expression studies were made with tissues harvested soon after experimental feeding to examine the early changes associated with TD. Thus, studies on alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP) and glutathione, as well as reverse transcriptase-polymerase chain reaction (RT-PCR) assays, were conducted on growth plate tissues from chickens aged 10 days, harvested within 2 h of the cessation of thiram feeding. The assays described in the following sections were repeated in two separate experiments which showed similar trends. The results reported here are based on a trial with one set of birds.

ALP and TRAP Assays

ALP is a marker of calcification and TRAP indicates osteoclastic activity related to skeletal remodelling (Boyle *et al.*, 2003). Both ALP and TRAP activities were assayed in the same tissue extract. Growth plate cartilage samples, harvested in cylinders (4 mm) from eight chickens of each group, were weighed and homogenized individually in ice-cold 0.15 M NaCl, 3 mM $NaHCO_3$ buffer, pH 7.4, and centrifuged at 10 000 ***g*** for 10 min at 4 °C. Both ALP and TRAP activities were measured in the supernate with *p*-nitrophenyl phosphate (*p*-NPP) hydrolysis at 37 °C for 15 min in a 96-well microassay format. ALP was determined at pH 9.3 (Reddi and Sullivan, 1980) and TRAP at pH 5.5 in 0.05 M acetate buffer containing 100 mM Na-tartrate, with minor modifications of previously published procedures (Rath *et al.*, 1981, 1995). The reaction mixture consisted of 100 µl of buffer containing *p*-NPP 5 mg/ml to which 10 µl of diluted or undiluted tissue extract were added. Each sample was assayed in duplicate. After incubation the reaction was stopped by the addition of an equal volume of 1 N NaOH. The absorbance unit of the hydrolysis product was measured at 410 nm, normalized, and expressed per mg of tissue. The percentage change in the enzyme activities for each sample was calculated relative to an averaged absorbance value in the control group and evaluated statistically. To determine whether thiram could directly affect alkaline or acid phosphatase activity, control tissue extracts were preincubated with or without 10 µM thiram for 30 min and assayed for the enzyme activities.

Glutathione Determination

Glutathione maintains oxido-reductive homeostasis, which plays an important role in cellular physiology (Rana *et al.*, 2002). Depletion of intracellular glutathione causes disruption of cellular metabolism, leading to various disease processes including cell death (Cotgreave and Gerdes, 1998). In view of the possible interaction between thiram and glutathione, we measured the total and the oxidized glutathione concentrations in sulphosalicylic acid extracts of the growth plate tissues according to Tietze (1969), by means of glutathione reductase and NADPH recycling method described by Allen *et al.* (2000). Growth plate cartilages harvested as above from contralateral tibias of eight birds were used for these studies. The oxidized glutathione (GSSG) was measured after 2-vinyl pyridine masking of reduced glutathione (GSH) (Griffith, 1980). The results were expressed as nmoles of GSH or GSSG/mg wet weight of tissues. Total GSH concentration represents the cumulative value from both oxidized and reduced glutathione.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) of mRNA Expression

To compare the changes in the gene expression profiles, approximately 50 mg of growth plate cartilage were harvested as above from four birds in each of the control and thiram- treated groups. The tissues were individually homogenized in TRIzol reagent (Molecular Research, Cincinnati, OH, USA) to isolate total RNA, according to the manufacturer's instructions. RNA was subjected to treatment with a Turbo DNA-free™ DNAse (Ambion, Austin, TX, USA) and then quantified with a Ribogreen reagent (Molecular Probe, Eugene, OR, USA). The purified RNA was frozen in aliquots at −80 °C. RNA (2 µg from each sample) was subjected to reverse transcription with a RETROscript kit (Ambion). After optimizing the assay conditions and cycle-dependent linearity of the polymerase chain reaction (PCR) with a multiplex assay kit (Qiagen, Carlsbad, CA, USA), 1 µl of cDNA from each sample was subjected to simultaneous PCR amplification with two sets of primers, one for a "house-keeping" β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the other for a candidate gene associated with growth plate development and metabolism. Both type II collagen and aggrecan were used as the genes associated with prehypertrophic chondrocyte differentiation, whereas type X collagen, transglutaminase, and a runt-related transcription factor (Runx2), were used associated with cartilage hypertrophy and maturation (Linsenmayer *et al.*, 1998; Nurminskaya *et al.*, 2002; Stricker *et al.*, 2002; Karsenty, 2003; Kronenberg, 2003). Matrix metalloproteinase (MMP)-2 and -13 were used as the genes associated with cartilage remodelling (Nie *et al.*, 1998). Both MMP-13 and vascular endothelial growth factor (VEGF), which are pro-angiogenic (Zijlstra *et al.*, 2004), were used as the markers of angiogenesis as they are produced by hypertrophic chondrocytes (Fosang *et al.*, 1996; D'Angelo *et al.*, 2000). Both house-keeping and candidate genes were coamplified with a "hot start" Taq Polymerase Multiplex PCR Kit (Qiagen, Valencia, CA, USA) in a 200 gradient Peltier Thermal Cycler (MJ Research, Watertown, MA, USA) for 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. The primer sequences for different

Table 1
The primer sequences of β-actin and other genes associated with growth plate development

Target genes	*Primers*	*Accession no. (and reference)*	*Amplicon size (bp)*
β-Actin	(f) 5′CATCACCATTGGCAATGAGAGG3′ (r) 5′GAT TCA TCG TAC TCC TGC TTG CT3′	L08165	273
GAPDH	(f) 5′AAGGTCATCCCAGAGCTGAA3′ (r)5′ACCATCAAGTCCACAACACG3′	V00406 (Daston *et al.*, 1996)	350
Aggrecan	(f) 5′TGTGAGGAAGGCTGGATCAAG3′ (r) 5′TGCTCACCCCTCGCCCCG3′	M88101 (Maeda and Noda, 2003)	661
Collagen II	(f) 5′TTAAAGATGTTGTAGGACCCCGAG3′ (r) 5′CGCAAAGTTTCCACCAAGTCC3′	M74435 (Nah and Upholt, 1991)	206
Collagen X	(f) 5′ATTGCCAGGGATGAAGGGACATAG3′ (r) 5′AGGTATTCCTGAAGGTCCTCTTGG3′	M13496	439
TGMse	(f) 5′GCCGCTACCGCCTGACACTG3′ (r) 5′AGCGCTTGCCACCCATCGTATCC3′	U47273	736
Runx2	(f) 5′TAAAGGTGACGGTGGATGG3′ (r) 5′TGTGGATTAAAAGGACTTGGTG3′	AF445419	190
MMP-2	(f) 5′AAACCCCGCTGTGGTAACCCCGATG3′ (r) 5′AGGGCTGTCCATCACCTATGCCACC3′	NM204420 (Nie *et al.*, 1998)	578
MMP-13	(f) 5′CAACCCAAAACATCCCAAAAC3′ (r) 5′CCATTCATAGCCCAAACCTTC3′	AF070478	258
VEGF	(f) 5′GGAAGCCCAACGAAGTTATC3′ (r) 5′AACCCGCACATCTCATCAG3′	AY168004	153

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TGMse, transglutaminase; Runx2, runt-related transcription factor; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; f, forward; r, reverse.

genes (Table 1) were obtained from published literature or designed with Primer Express software (Applied Biosystems, Foster City, CA, USA). The nucleotide probes were synthesized by Invitrogen (Carlsbad, CA, USA). The annealing temperature for all primers, including β-actin, was set at 60 °C. Preliminary experiments demonstrated that under these conditions all genes showed a linear increase in signal, depending upon the number of cycles and the cDNA concentrations. The PCR products were diluted 10-fold with water and analysed with a P/ACE 5500 capillary electrophoresis system equipped with a laser-induced fluorescence (LIF) detector (Beckman-Coulter, Fullerton, CA, USA) by means of procedures described earlier (Richards and Poch, 2002; Rath *et al.*, 2003). A capillary filled with a 70% concentration of DNA gel buffer (Sigma Chemical Co., St Louis, MO, USA) containing a DNA intercalating dye Enhance™ (Beckman-Coulter) was used to develop the electropherogram. The samples were injected electrokinetically at 3 kV for 10 s at the cathode end of a 75-μm internal diameter μSIL DNA capillary (Agilent Technology, San Jose, CA, USA) and separated at a constant current of 15 kV for 5 min. In between separations the capillary was rinsed for 1 min with methanol followed by a 1-min rinse with a fresh gel buffer. The total separating distance from the inlet to the detector was 18 cm. The PCR products were detected as fluorescent peaks. The relative changes in the expression of different genes were calculated by dividing the peak areas of the candidate gene by the β-actin peak areas. The average expression was calculated from the results of four individual samples per group. The sizes of the amplicons were determined from a standard curve obtained from a low mass DNA ladder (Invitrogen). The coefficient of variation (CV) for molecular size estimates of different amplicons obtained by the capillary electrophoresis method was within 5–10% of the range of the predicted size.

Bromodeoxyuridine (BrdU) Incorporation

To determine whether thiram induced proliferation of transition zone chondrocytes leading to the broadening of the growth plate, BrdU labelling was used to examine growth plate cartilages harvested from control and thiram-fed chickens aged 11 and 14 days. In these birds there was a visual difference in the width of growth plates between the control and thiram-treated groups, the latter showing considerable broadening. Cylindrical explants (1 mm diameter) of growth plate cartilage from four chickens in each group were each incubated in 1 ml of complete Dulbecco's modified Eagle's medium (DMEM) containing

antibiotics, fetal bovine serum 1%, and BrdU 100 µg /ml for 6 h, washed in 0.9% saline, and fixed in 4% *p*-formaldehyde. At the end of incubation, the tissues were processed for histology as above and the sections were immune-stained with BrdU detection reagents (Zymed Laboratories, South San Francisco, CA, USA) according to the manufacturer's instructions. The final step of colour development was made with a Histomark Kit (KPL, Gaithesburg, MD, USA), the horseradish peroxidase (HRP) component of which yielded a blue colour in BrdU labelled cells. The labelling of cells in various regions of the growth plates was evaluated qualitatively by photographing the sections under a microscope. At least two sections from each group were examined for the distribution of labels.

Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-end Labelling (TUNEL)

To demonstrate apoptosis, longitudinal sections of growth plates from the left tibia of four chickens in each group on each of four occasions (at ages 10, 11, 15 and 22 days) were fixed in neutral buffered formalin for 48 h, followed by decalcification with Poly no-Cal decalcifying solution (Polyscience, Warrington, PA, USA) and embedded in paraffin wax for routine histological procedures. Sections (5 µm) of growth plate tissues were used for TUNEL assay (Ben-Sason *et al.*, 1995) with fluorescein-12 dUTP as the label (Rath *et al.*, 1998). Tissue sections were treated with proteinase K, preincubated in equilibration buffer containing 30 mM Tris, 140 mM Na cacodylate buffer and 1 mM $CoCl_2$, pH 7.2, and then labelled in the same buffer containing 1 mM DTT, TdT 0.5 U/µl, and 10 nM fluorescein-12 dUTP for 1 h at 37 °C in a humidified chamber. The reaction was stopped with 300 mM NaCl/30 mM Na citrate, pH 8.0 containing bovine serum albumin 0.05%. After washing, the sections were counterstained with propidium iodide (PI), mounted in Vectashield mounting medium, examined with an Olympus BX-50 fluorescent microscope, and photographed with an Optronic digital camera. PI binding to the nucleus caused bright red fluorescence, whereas the incorporation of fluorescein-dUTP produced yellow to green fluorescence in cells undergoing apoptosis (Rath *et al.*, 1998).

DNA Fragmentation

DNA fragmentation, a consequence of apoptotic cell death, was assessed from DNA extracted from approximately 200 mg of growth plate tissues pooled from four birds in each group on three occasions (at ages 10, 15, and 22 days) with DNAzol reagent (Molecular Research, Cincinnati, OH, USA). After determining the concentrations of DNA by means of a Sybr Green I dye binding assay (Molecular Probe), approximately 5 µg of DNA were loaded into QIAquick spin columns (Qiagen, Valencia, CA, USA) and the DNA was eluted from the columns according to the manufacturer's instructions. DNA (25 ng) derived from each control or thiram-fed growth plate sample was subjected to electrophoresis in 1.4% agarose and stained with Sybr Green I dye, and the bands were "visualized" by means of the Gel Doc system (BioRad, Hercules, CA, USA) and photographed. A low mass DNA molecular weight standard was used as marker.

Statistics

Quantitative results were expressed as mean ± SEM; the differences between the two groups were analysed and compared by Student's *t* test (SAS software; SAS Institute, Cary, NC, USA). Values of $P<0.05$ were considered significant.

Results

Characterization of TD

Thiram-fed chicks showed bilateral TD, more than 95% of birds being affected by 16 days of age. Whereas the control birds showed a TD index of 0.13 ± 0.07 ($n=24$), the thiram-treated chickens had an index of 1.97 ± 0.08 ($n=16$) (Fig. 1). Histopathology showed many post-proliferative chondrocytes exhibiting diminished cytoplasm and nuclear volume, and atrophying blood vessels, in the TD-affected growth plate (not shown).

Biochemical Assays

Table 2 shows that ALP activity of growth plate homogenates was similar in control and thiram-fed birds, but the TRAP activity was reduced in the latter. Direct incubation of tissue extracts with 10 µM thiram caused no differences in the enzyme activities (not shown). A significant reduction in total glutathione (GSH), and in the ratio of oxidized glutathione (GSSG) to total glutathione was evident.

RT-PCR

Fig. 2 shows typical separation profiles of different PCR products generated when two different sets of primers consisting of a housekeeping and a candidate gene were subjected to RT-PCR

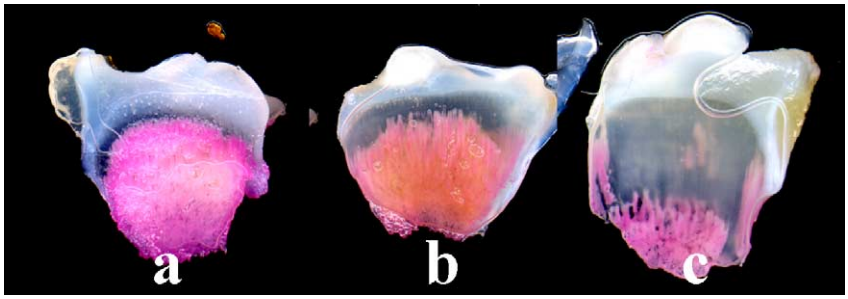

Fig. 1. Typical morphology of growth plates from control and thiram-fed chickens aged 16 days. (a) Control growth plate. (b) Growth plate with TD severity score of 1 from a thiram-fed chicken. (c) Growth plate with a severity score of 2 from a thiram-fed chicken.

Table 2
Changes in biochemical variables of growth plate cartilage from 10-day-old chickens fed control or thiram supplemented diet ($n=8$)

Chickens	*Alkaline phosphatase (% activity)±SEM*	*Tartrate-resistant acid phosphatase (% activity)±SEM*	*Total GSH (nmoles/mg)±SEM*	*GSSG:GSH*
Control	100±8	100±27	0.45±0.03	0.102±0.01
Thiram-fed	109±9	44±3*	0.29±0.02*	0.071±0.01*

*Significantly different from control value ($P<0.05$).

simultaneously with different aliquots of cDNA from a control bird. Table 3 shows the changes in the expression profiles of different genes in relation to β-actin in the growth plates of control and thiram-fed birds. There were no significant differences in the expression of aggrecan, type II collagen, type X collagen, glyceraldehyde 3 phosphate dehydrogenase (GAPDH), transglutaminase 2 (TGMse), Runx2, or matrix metalloproteinase(MMP)-2, but the expression of MMP-13 and of VEGF was increased in thiram-fed chickens (Table 3).

BrdU Incorporation

There was no incorporation of BrdU into the post-proliferative prehypertrophic or hypertrophic chondrocytes in either control or thiram-treated birds aged 11 or 14 days. However, the chondrocytes in the proliferating region showed incorporation of BrdU at both ages; this appeared not to be affected by thiram, even when the growth plate was afflicted with TD on day 14 (Fig. 3).

Cell Death

At 10 days of age (i.e., 2 days after the beginning of feed treatment) there was little visual difference between the growth plates from control and thiram-treated chickens. However, endothelial cell apoptosis occurred in occasional capillary vessels in the transition zone of the growth plate cartilage of thiram-treated birds, as shown by fluorescein label. The apoptosis of capillaries had increased by the age of 11 days, at which time clusters of 3–4 chondrocytes showed nuclear fragmentation or nuclear condensation. The capillary vessels at 10 and 11 days of age were surrounded by apparently normal chondrocytes (Fig. 4a, b). By the age of 15 days there were many blood capillaries and

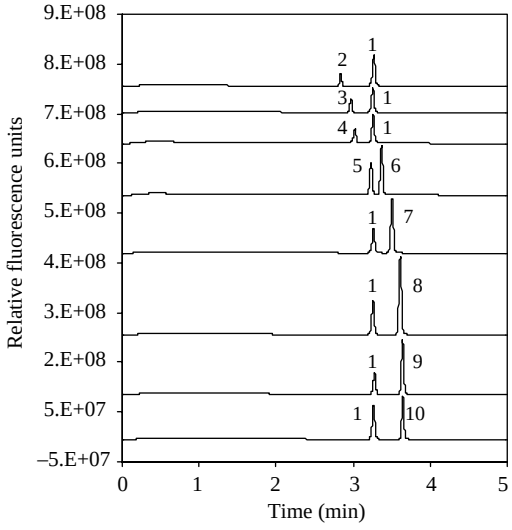

Fig. 2. Electropherogram profiles of PCR products of a typical sample of a control growth plate cDNA. The numbers adjacent to peaks correspond to genes amplified. 1, β-actin; 2, VEGF; 3, RUNX2; 4, collagen II; 5, glyceraldehyde 3-phosphate dehydrogenase; 6, matrix metalloproteinase-13; 7, collagen X; 8, MMP-2; 9, aggrecan; 10, transglutaminase-2.

Table 3
Comparative changes in the expression of candidate genes in growth plates of 10-day-old control or thiram-fed chickens ($n=4$). The numbers correspond to the ratios of peak areas of corresponding genes to β-actin ± SEM

	Result in*	
Target genes	*control chickens*	*thiram-fed chickens*
Aggrecan	1.90 ± 0.15	2.25 ± 0.09
Collagen II	0.49 ± 0.06	0.60 ± 0.02
Collagen X	2.16 ± 0.05	2.35 ± 0.15
GAPDH	1.35 ± 0.19	1.23 ± 0.08
TGMse	0.93 ± 0.11	1.10 ± 0.22
Runx2	0.54 ± 0.07	0.38 ± 0.08
MMP-2	1.80 ± 0.37	0.97 ± 0.35
MMP-13	0.86 ± 0.05	0.99 ± 0.01†
VEGF	0.34 ± 0.03	0.59 ± 0.02†

*Ratio of peak area of gene to that of β-actin.
†Significantly different from control value ($P \leq 0.05$).

chondrocytes in the hypertrophic zones of the growth plates of thiram-treated birds; these showed apoptotic changes with massive cell death by the age of 22 days (Fig. 4 c, d). Histological examination of growth plates from thiram-treated birds showed chondrocytes with nuclear shrinkage and dehydration, and empty cartilage lacunae. DNA fragmentation analysis showed a progressive increase in DNA breakage, supporting the morphological apoptotic data (Fig. 5).

Discussion

The objective of the present study was to examine the changes in the growth plate induced by thiram, which leads to the development of TD. BrdU labelling showed that the broadening of growth plate cartilage was not due to an increased multiplication of chondrocytes in the post-proliferative zone, as suggested by Pines *et al.* (1998). The present results accord with those of Gay and Leach (1985) and Farquharson *et al.* (1992), who showed that the chondrocytes in the TD lesions did not increase in number. Moreover, thiram appeared to have no discernible effect on the proliferative zone chondrocytes, which showed BrdU labelling comparable with that in control birds, even though the growth plates were affected with TD. ALP, an important mediator of cartilage calcification, was down-regulated in TD-affected cartilage which failed to calcify (Knopov *et al.*, 1997); however, Farquharson *et al.* (1992) observed increased ALP activity in growth plates in which TD had been induced by feeding a diet low in Ca and vitamin D_3. While the contradictory results between these two studies may have been due to the use of different experimental models, our results with thiram indicated that, at least during the early stage of TD, the ALP activity of the growth plate was not affected. TRAP, on the other hand, which is produced by different skeletal resorbing cells such as osteoclasts, chondroclasts and macrophages (Suda *et al.*, 1992), was reduced in the growth plates of thiram-treated birds. Rath *et al.* (1995) showed that the in-vitro treatment of epiphyseal chondrocytes with thiram resulted in reduced acid phosphatase activity. However, it is not known whether thiram interferes with the generation of the cells responsible for cartilage resorption or only impairs their function. Direct addition of thiram to

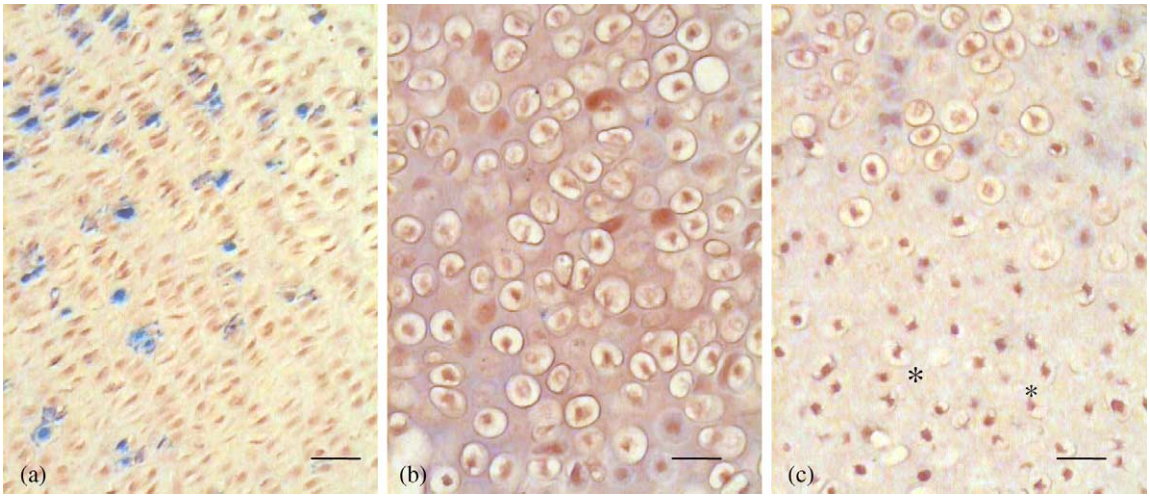

Fig. 3. BrdU incorporation into the growth plate chondrocytes of 14-day-old control or thiram-fed chickens. (a) Proliferative zone cartilage of thiram-fed chicken growth plate, showing incorporation of BrdU into chondrocytes. (b) Growth plate with hypertrophic chondrocytes from a control chicken. (c) Transition zone chondrocytes in the growth plate of a thiram-fed chicken, showing no BrdU incorporation and many shrunken chondrocytes (*). Bar, 10 μm.

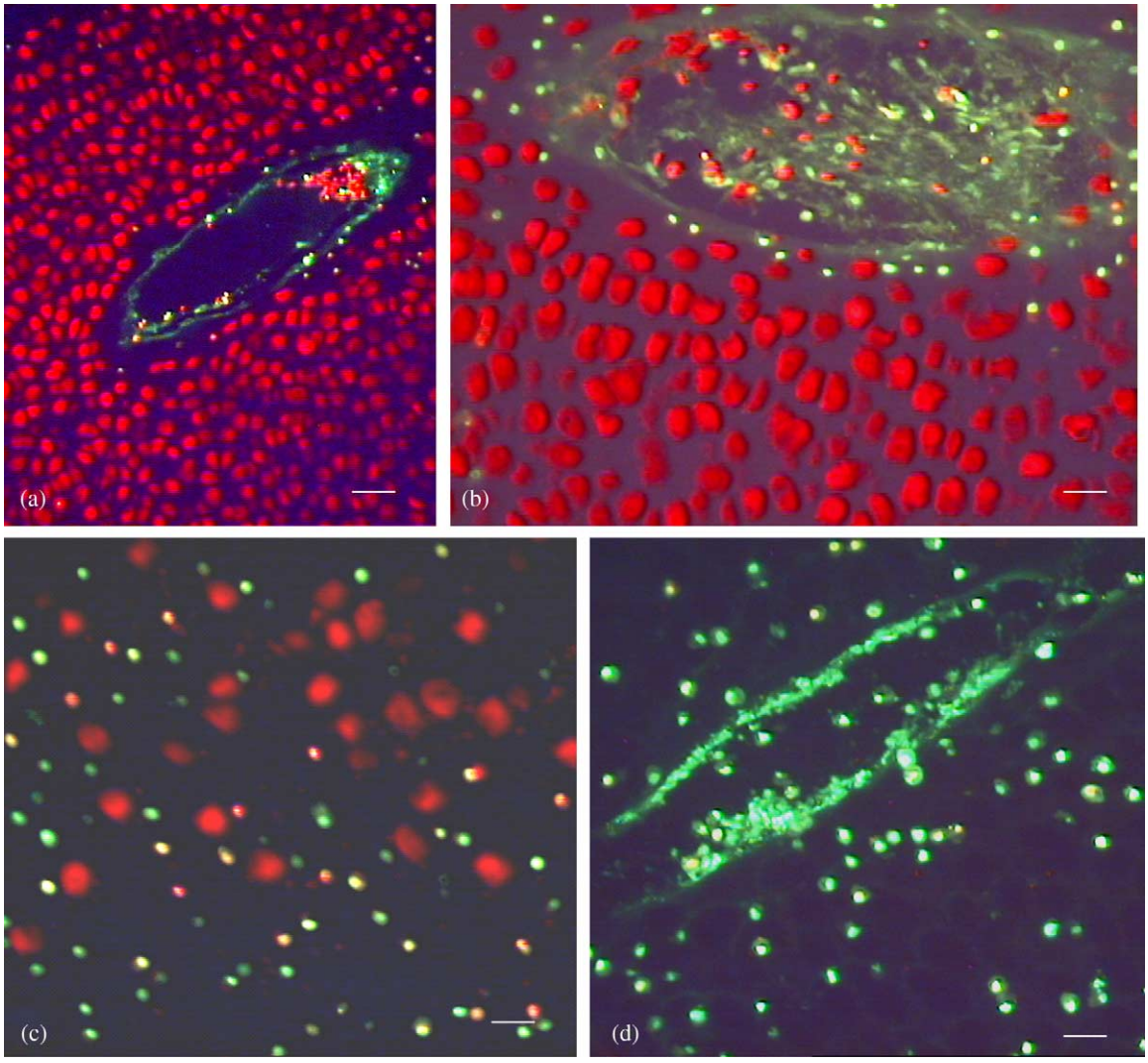

Fig. 4. TUNEL labelling of hypertrophic zone of growth plates from thiram-fed chickens. Post-proliferative zone chondrocytes are shown in birds aged (a) 10 days, (b) 11 days, (c) 15 days, or (d) 22 days. Green corresponds to fluorescein labelled apoptotic nuclei. Bars, 25 μM (a) and 10 μM (b, c, d).

tissue extract was insufficient to suppress TRAP activity. Chondroclast failure as a cause of TD was suggested by Lawler *et al.* (1985). Regardless of the mechanism, thiram appears to interfere with the cartilage turnover process, contributing to its retention. The effect of thiram on glutathione concentration in the growth plate was examined because of the likelihood that thiram would react with thiol groups and interfere with glutathione metabolism. Glutathione plays an important role in the maintenance of cellular homeostasis, the depletion of which may lead to pathological changes, including cell death (Elskens and Penninckx, 1997; Cotgreave and Gerdes, 1998; Balakirev and Zimmer, 2001; Rana *et al.*, 2002). Thiram treatment caused reduction in both total and oxidized glutathione. The effects of thiram on skin fibroblasts in culture were demonstrated by Cereser *et al.* (2001), who reported an increase in oxidized glutathione with a concomitant depletion in reduced and total glutathione (GSH), suggesting that thiram is an inducer of oxidative stress and cytotoxicity; in the present experiment, however, there was no increase in the oxidized glutathione concentrations. Disulphiram, an ethyl analogue of thiram, also causes mitochondrial injury through its effects on GSH metabolism (Balakirev and Zimmer, 2001). Taken together, these results indicate that thiram may affect growth plate metabolism through its anti-metabolic and cytotoxic effects.

It has been suggested that prehypertrophic chondrocytes fail to undergo maturational changes such as hypertrophic transition, a step crucial to

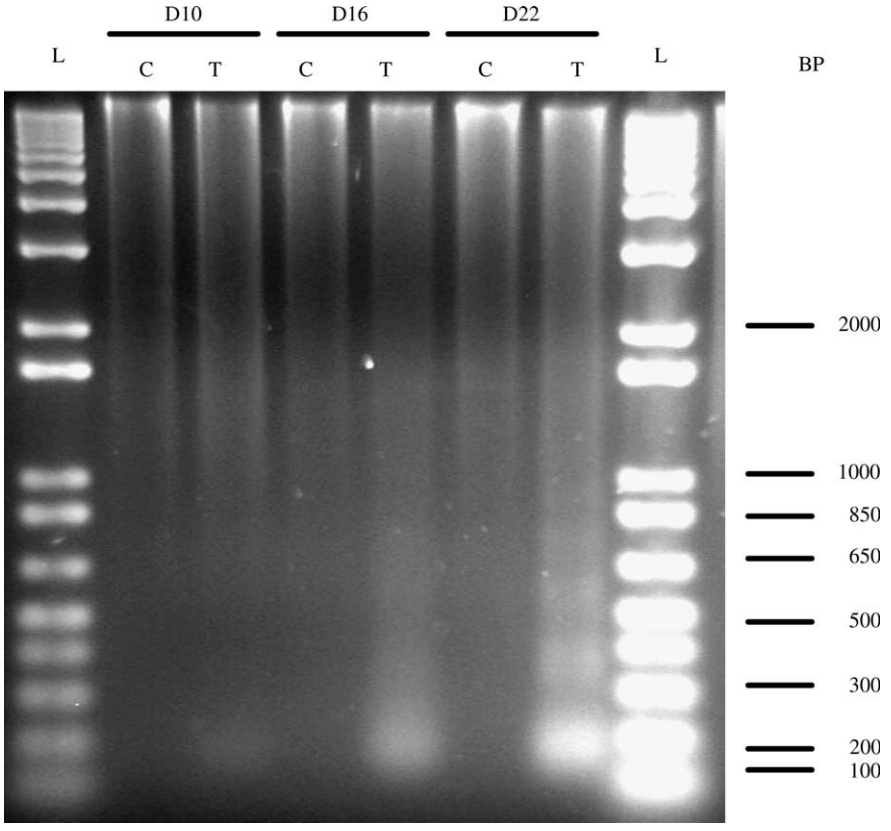

Fig. 5. DNA fragmentation in growth plate of chickens aged 10 days (D10), 16 days (D16) or 22 days (D22), having been fed a control (C) or thiram (T) diet. BP, base pair; L, DNA ladder.

endochondral bone formation, and thus remain undifferentiated (Praul *et al.*, 2000; Farquharson, 2002). Conversely, dedifferentiation of hypertrophic chondrocytes to the prehypertrophic phenotype has also been suggested as a mechanism for the accumulation of non-hypertrophic chondrocytes in TD-affected growth plates (Farquharson, 2002). In either case, chondrocyte maturation-related gene expression is likely to be affected, to maintain the prehypertrophic state of the chondrocytes. To examine the possible effect of thiram on the genes associated with growth plate development and maturation, we analysed the expression of type II collagen and aggrecan genes associated with undifferentiated prehypertrophic chondrocytes, which on hypertrophic differentiation switch to the production of type X collagen (Linsenmayer *et al.*, 1998). As well as type X collagen, other maturation markers of chondrocytes include transglutaminase 2, (TGMse), which is also associated with hypertrophy and terminal differentiation (Nurminskaya *et al.*, 2002), and a runt-related transcription factor (Runx2) produced by chondrocytes undergoing hypertrophy (Stricker *et al.*, 2002; Karsenty, 2003; Kronenberg, 2003). There was no change in the expression of either aggrecan, collagen (type II or type X), TGMse or Runx2 mRNA, suggesting that thiram does not impair the maturational processes. Matrix metalloproteinases (MMPs) play a role in the breakdown of extracellular matrix, initiating tissue remodelling and angiogenesis. Both MMP-2 and -13 are expressed in avian growth plate, MMP-13 being produced by hypertrophic chondrocytes (Blair *et al.*, 1989; Wardale and Duance, 1996; Nie *et al.*, 1998; D'Angelo *et al.*, 2000). Rath *et al.* (1997) demonstrated a severe reduction in MMP-2 activity in TD-affected growth plate cartilage. However, in the present study no changes in the expression of MMP-2 were found; on the other hand, the expression of MMP-13 was increased in the growth plates of thiram-treated birds. MMP-13 (collagenase-3), which has both aggrecanase and type X collagenase activity (Fosang *et al.*, 1996; D'Angelo *et al.*, 2000) is also pro-angiogenic (Zijlstra *et al.*, 2004). As with MMP-13, there was an increase in VEGF gene expression. VEGF, which is a principal mediator of angiogenesis (Folkman and D'Amore, 1996), is produced by hypertrophic chondrocytes (Gerber *et al.*, 1999; Carlevaro *et al.*, 2000; Kronenberg, 2003).

Because the TD cartilage plug is avascular, the paradoxical increased expression of the pro-angiogenic genes MMP-13 and VEGF in thiram-treated growth plates was intriguing. Experimental interference with angiogenesis by anti-angiogenic chemicals prevented chondrocyte maturation in chicken limb buds (Yin *et al.*, 2002). However, it is not known whether the increased expression of these pro-angiogenic mRNA genes reflects an increased production of the corresponding proteins that are eventually responsible for angiogenesis. Conversely, if such proteins were produced it would still necessitate the presence of intact capillaries and endothelial cells to respond to these factors, thereby causing growth plate angiogenesis. The TUNEL results showed apoptosis of the endothelial cells outlining blood capillaries as early as 10 and 11 days of age, implying that the vascular endothelial cells were probably the first targets to be affected by thiram and to undergo apoptosis; this was followed by the death of transitional zone chondrocytes, culminating in massive cell death in the distal regions of growth plate by 22 days of age. Rath *et al.* (2004) demonstrated extensive histological changes in the growth plate accompanying the regression of blood capillaries in fully developed TD lesions. Apoptosis of growth plate cells was supported by DNA fragmentation results, which showed a progressive increase in DNA laddering. In the light of this observation, it would appear that in spite of the expression of most maturational markers, including increased expression of both MMP-13 and VEGF genes, the growth plate still fails to undergo complete development due to the death of blood vessels and chondrocytes. The extent of damage possibly determines the severity of TD.

The anti-angiogenic activity of thiram and its ethyl analogue disulphiram, has been shown in cancer tissues (Marilkovsky, 2002; Shian *et al.*, 2003). Unlike its mammalian counterparts, the avian growth plate, including the transitional zone, is a well vascularized tissue (Loveridge *et al.*, 1992). Destruction of existing capillaries and prevention of angiogenesis by the action of thiram would therefore be expected to arrest local development of the growth plate. Experimental ablation of metaphyseal vessels produced TD-like defects in chickens (Riddel, 1975). Prevention of angiogenesis, together with chondrocyte death, appears to result in the TD lesions and a secondary down-regulation of most genes and proteins (Farquharson, 2002).

The molecular mechanism of cell death induced by thiram, however, is not understood. Depletion of glutathione, leading to cellular stress, may play a role. Additionally, the dithiocarbamates, particularly pyrollidine dithiocarbamate (PDTC), inhibit NFkB, a transcription factor involved in the regulation of a variety of physiological processes including cell survival (Wang *et al.*, 2003). NFkB also regulates osteoclast differentiation (Boyle *et al.*, 2003). Whether thiram prevents NFkB activation, leading to the impairment of osteoclast generation and promoting endothelial cell and chondrocyte apoptosis, is not known. Rath *et al.* (2004) showed that PDTC when fed to chickens induced TD.

Finally, it is unclear why post-proliferative chondrocytes, particularly the maturing chondrocytes, are susceptible to thiram. We reason that the process of hypertrophy requires substantial changes in cell volume (Hunziker *et al.*, 1987; White and Wallis, 2001), which may require compensatory changes in membrane fluidity. Because thiram is hydrophobic, it is possible that its incorporation into membranes is facilitated at this stage, damaging cellular mechanisms including those associated with mitochondria, and precipitating cell death.

In conclusion, this study showed that thiram did not affect chondrocyte proliferation or chondrocyte maturation-related gene expression; however, it depleted glutathione levels and induced death of capillary blood vessels and transition zone chondrocytes. This caused impaired cartilage resorption and further growth plate development, resulting in an avascular plug of non-viable cartilage.

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